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TITLE: A Model for Understanding the Genetic Basis for Disparity in Prostate Cancer Risk

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14. ABSTRACT
Prostate cancer is the most commonly diagnosed cancer in men. Among African American men, the incidence of prostate cancer is approximately 60% higher and the mortality rate in this population is 2 to 3 times greater compared with European American men. The reasons for this disparity are not completely understood. Current tools in hand to study these differences, such as genetically altered mouse models, are useful for dissecting roles of specific genes and signaling pathways in intact animal, but have limited utility for understanding differences in disease susceptibility in humans.
The overall objective of this application is to model prostate epithelial cells to understand the molecular basis for the disparities in prostate cancer risk between white Caucasian and black African-American men. The specific aims are: 1) to establish conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSC into cells with characteristics of prostate epithelium, 2) identify differences in gene expression and epigenetic signatures between prostate epithelial cells derived from iPSC of Caucasian and African-American foreskin fibroblasts and 3) compare and establish methods to transform differentiated prostate epithelial cells to identify differences in susceptibility to transformation.

15. SUBJECT TERMS

Nothing listed

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Table of Contents

Page

RPPR format for DOD progress report

The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. **INTRODUCTION:**

   Prostate cancer is the most commonly diagnosed cancer in men in Europe and the United States. Numerous studies have indicated genetics to have a major role in the etiology of this disease; as much as 42% of the risk may be explained by heritable factors. Moreover, among African American men, the incidence of prostate cancer is approximately 60% higher and the mortality rate in this population is 2 to 3 times greater compared with European American men The reasons for this disparity are not completely understood. Since no clear patterns were observed for association with dietary factors or life style factors such as physical activity, occupational history, sexual behavior and other health conditions), it is likely that inherent genetic and epigenetic differences, presumably both germ-line and prostate-cell specific, contribute to this disparity in prostate cancer risk. Efforts are ongoing to identify molecular mechanism and common risk alleles for prostate cancer risk using genome-wide association studies. While identification of individuals/population at risk is important, additional in-depth studies are needed to understand the genetic and molecular mechanisms responsible for the differences in susceptibility of prostate epithelial cells to malignant transformation. However, limited access to human prostate tissue prior to onset of age-related or malignant changes has hampered analyses of genetic and epigenetic mechanisms intrinsic to prostate epithelial cells. More recent strategies to study prostate development, maturation and carcinogenesis included differentiation of human embryonic stem cells (hESC) using rodent mesenchyme. Studies using hESC also have many limitations including ongoing ethical debate and the number of available cell lines, especially that represent different genetic ancestry. Induced pluripotent stem cells (iPSC) offer a useful alternative to hESC. For example, recently, in vivo regeneration potential of human iPSC has been documented. The proposed project is aimed to test the hypothesis that differentiation of neonatal foreskin fibroblasts-derived iPSC to prostate epithelial cells is a unique and powerful strategy to investigate the genetic and molecular basis for the disparities in prostate cancer risk among men of different genetic ancestry.

2. **KEYWORDS:**

   Induced Pluripotent cells, Directed differentiation, Prostate Cancer, Disparity in Cancer Risk, African-American

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

     - To establish culture conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSC into cells with characteristics of prostate epithelium.
     - Identify differences in gene expression and epigenetic signatures between prostate epithelial cells derived from iPSC of Caucasian and African-American foreskin fibroblasts.
     - Compare and establish methods to transform differentiated prostate epithelial cells to identify differences in susceptibility to transformation

   - **What was accomplished under these goals?**

     - **See below**

Task 1.

A) Embryoid body formation of iPS cells:

In the previous project report, we demonstrated that the iPS cells generated displayed three-germ layer differentiation into ectoderm, mesoderm and endoderm. In the present report, we report characterization of these cells by embryoid body formation assays. We did this additional characterization because our goal is not just to develop prostate cells from iPS cells, but also the possibility of 3D prostate organoids. Therefore, the formation of embryoid body is an important feature required for 3D organoid formation *in vitro*.

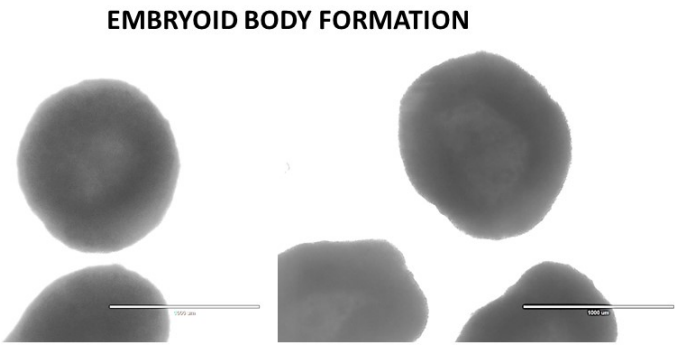

Figure 1. iPSC display Embryoid body formation. The assays showed that the iPSCs we generated exhibit capacity to form embryoid bodies (Figure 1). Altogether, these data and 3-germ layer differentiation assays support that the iPSCs reprogramming was successful and the iPSCs generated display pluripotent stem cell features.

B) Chromosomal analysis of iPSC clones (karyotyping):

iPSC clones were analyzed by WiCell's Cytogenetics laboratory to determine their karyotypes. 50 % of the clones displayed normal karyotyping. 2 African and 2 European clones with normal karyotypes were selected for expansion and prostate organoid differentiation (**Figure 2**). More clones will be prepared for karyotyping and future analysis.

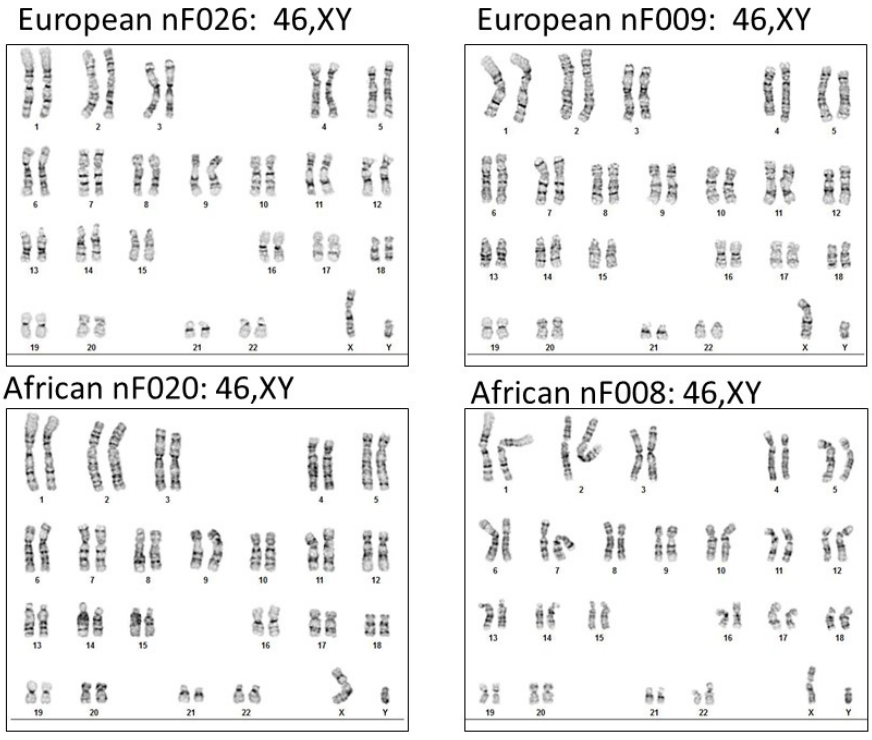

Figure 2. Karyotyping Analysis of iPSC clones. Two European fibroblasts-derived iPSC clones and two African fibroblasts-derived clones displaying normal karyotypes as determined by WiCell. Pictures depict the number and structure of chromosomes performed with G-band staining.

C) Establishment of optimal conditions for Prostate organoids differentiation *in vitro*:
Conditions for prostate organoids differentiation from iPSCs were tested using three approaches during optimization. First, we used (1) Induction of Prostate Organoids with Stromal and UGSM feeder cells; (2) Prostate Stepwise Differentiation; and (3) Prostate Direct Differentiation. The methods and results are described below:

C.1) Induction of Prostate Organoids with Stromal and UGSM feeder cells:
We co-cultured iPS cells with human neonatal fibroblasts (hNF) and mice neonatal urognenital sinus mesemchyme (UGSM) in prostate epithelial cell medium. Fibroblasts are cells from the prostate's stromal structure and they secrete components of the extracellular matrix (ECM) and basement membrane that may have inductive roles for prostate growth, development and differentiation. Additionally, it is well known that the prostate development and differentiation rises from the embryonic UGSM bud. We therefore hypothesized that co-culture with stromal and UGSM feeder cells in prostate epithelial medium could induce prostate cells in 2D organoids *in vitro* (Figure 3). Prostate differentiation results indicated that iPSCs were able to differentiate and organize themselves into 2D organoids with some prostate features and also expressed CK18, NKX3.1 and AR, markers of prostate cells, as determined by immunofluorescence (data not shown). We also determined that conditioned medium from fibroblasts and UGSM generated similar results. Unfortunately, after several trials we found that using these methods were laborious and the stability of the organoids generated was variable, being affected by several factors such as quality of the inductive cells such as passage of the UGSM and fibroblasts. For instance, freshly isolated UGSM was more efficient to induce differentiation but after passage 4 or freeze-thawed UGSM resulted in delayed differentiation. We therefore, set up to find prostate differentiation under defined conditions without inductive feeder cells co-culture or conditioned medium.

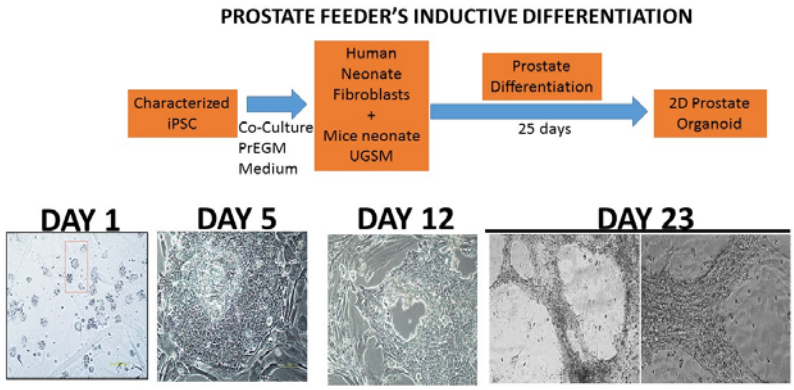

Figure 3. Induction of Prostate Organoids with Stromal and UGSM feeder cells. Top panel depicts the general protocol for prostate *in vitro* differentiation using the inductive method by co-culturing human neonate fibroblasts and mice neonate UGSM cells. Lower panel shows the progressive morphological changes indicative of prostate organoid differentiation. Day 1 shows the single cell suspension and days 5 – 12 display colonies with formation of luminal-like structures. Day 23 shows larger luminal-likes structures and formation of more differentiated tissues.

C.2) Prostate Stepwise Differentiation:
The embryonic origin of the prostate gland fate and organogenesis is known to rise from the urogenital sinus from the endoderm layer. We therefore, tested a directed differentiation protocol using signaling growth and differentiation molecules; and specialized prostate epithelial and stromal cell media (Top panel, **Figure 4**). iPSC cells were derived to definitive endorderm with activin A for three days. Endoderm differentiation was verified by expression of SOX17, as indicated by IF. Following endoderm differentiation, endoderm-differentiated cells were treated with WNT10B and FGF10A for 4 days to drive them into prostate fate. Organoids were differentiated into prostate for 30 days by culturing in prostate differentiation medium prepared with prostate epithelial cell growth media (PrEGM-LONZA) and stromal cell growth media (SCGM-LONZA) in 1:2 proportions, respectively. PrEGM was prepared with bovine pituitary extract (BPE), Human Insulin, hydrocortisone, gentamicin sulfate amphotericin-B, retinoic acid, transferrin, triiodothyronineT3,

Epinephrine and human EGF as indicated by the medium's manufacturer. SCGM was prepared with 5% FBS, human FGF-B, human insulin and gentamicin sulfate amphotericin-B, according to the manufacturer's recommendations. In addition to the PrEGM/SCGM mixture, prostate differentiation medium was supplemented with 1X B27, Noggin, EGF, R-Spondin1, dihydrotestosterone (DHT), 2 mM of L-glutamine, 1% penicillin-streptomycin and 15 mM HEPES. Results indicated that iPS cells displayed morphological changes indiciative of differentiation process by forming outgrowth and path finding projections the first two weeks. However, no significant morphological changes were seen in the following third and fourth weeks (Figure 4, lower panel).

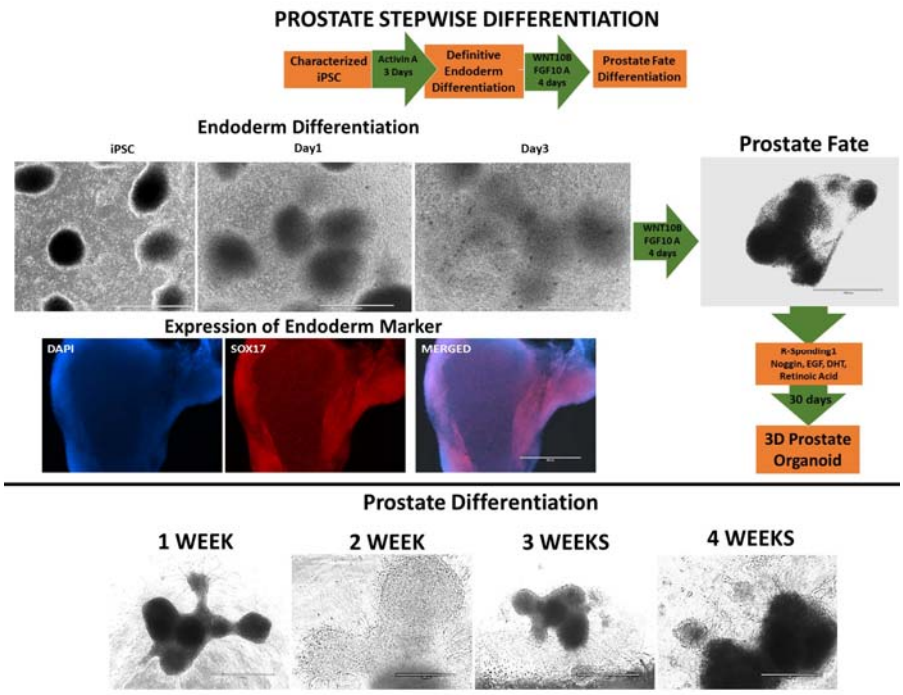

Figure 4. Prostate Stepwise Differentiation. iPS cells were differentiated into endoderm cells by culturing them in ACTIVIN A and verified by expression of endoderm marker SOX17. Endoderm differentiated cells were differentiated into prostate cell fate by incubation with WNT10B and FGF10A for 4 days. We followed the prostate differentiation process as indicated and it showed some morphological changes with formation of projections outgrowth/pathfindings buds. However, after two weeks these organoids did not show further significant changes and displayed no internal branching or morphological features indicative of functional prostate differentiation.

C.3) Prostate Direct Differentiation:

The third protocol, involved the direct differentiation of iPSCs using prostate differentiation medium (as described in the stepwise method), without endoderm and prostate fate transitions (Figure 5). This protocol was run in 2D and 3D and organoids progressively showed morphological changes every week. After first week, cells started to multiply and organize themselves forming multiple semicircular shapes in 2D while 3D organoids formed some small outgrowth. The second week, organoids significantly enhanced their semicircular shape in 2D, and 3D organoids showed some preliminary changes, indicative of internal reorganization. The third week, 2D organoids showed multiple areas of enriched differentiation with semicircular shapes exhibiting internal branching and release of spheroid cells outside the structures with enriched differentiation/branching. 3D organoids also exhibited remarkable changes with internal branching and also releasing of spheroid cells to the medium, similar to prostaspheres (Figure 6).

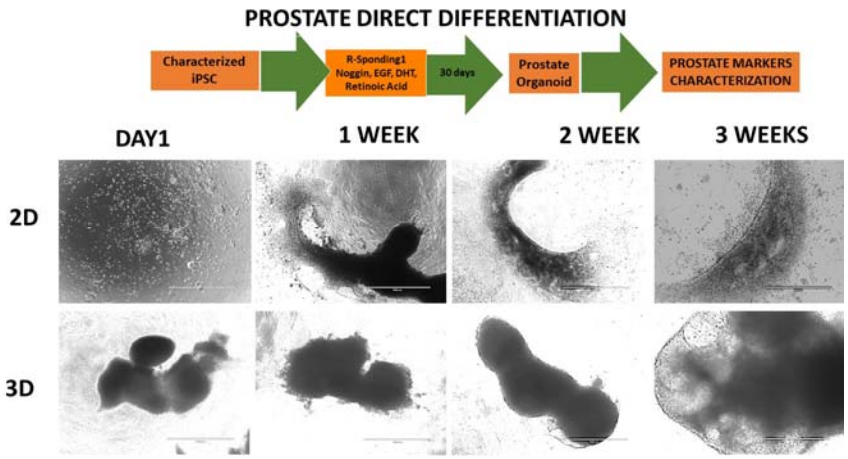

Figure 5. Prostate Direct Differentiation. This approach was much more efficient compared to the previous

two methods. Cells were able to multiply and organize themselves under these conditions generating prostate organoids in both 2D and 3D. Organoids displayed progressive morphological changes forming internal ducts and produced spheroid cells, similar to prostaspheres.

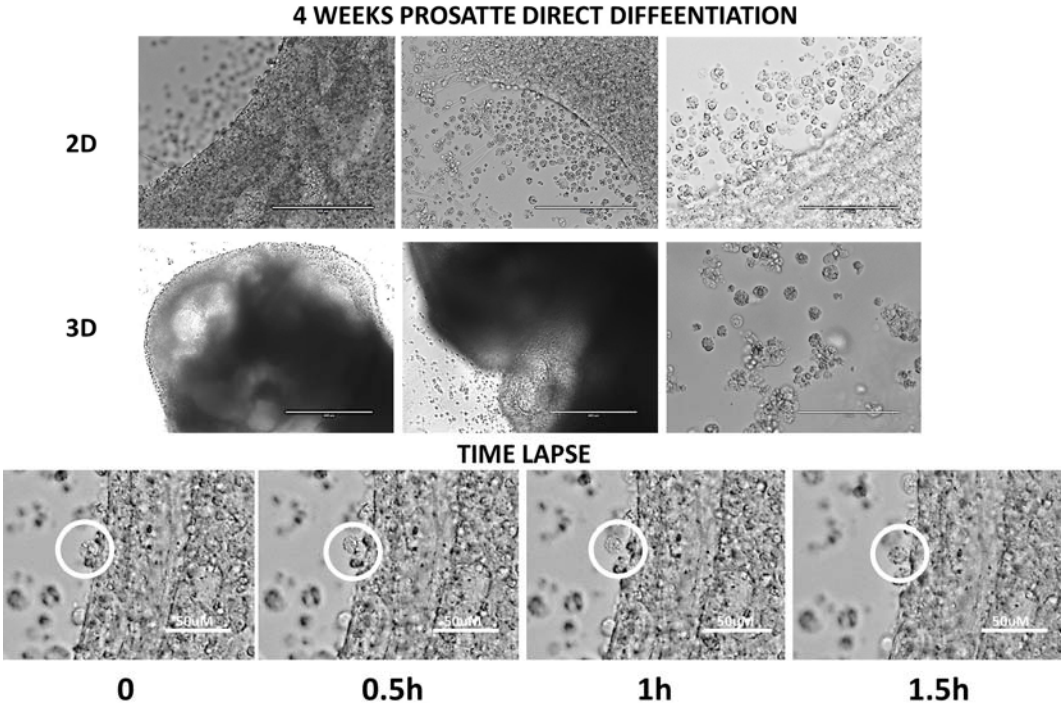

Figure 6. Organoids develop Prostate-related functional features. The fourth week, both 2D and 3D organoids displayed even more remarkable morphological differentiation showing release of spheroid cells and internal branching. Time-lapse of 2D organoids confirmed that the prostate organoids were generating these spheroid cells. These spheroid cells are similar to prostaspheres generated in other prostate organoid models from healthy prostate and prostate cancer tissues samples.

Importantly, together with morphological changes functionally associated with prostate, expression of prostate markers was detected at two weeks and progressively increased after four weeks after induction of prostate differentiation as determined by immunofluorescence (IF) of 2D organoids (Figures 6 and 7).

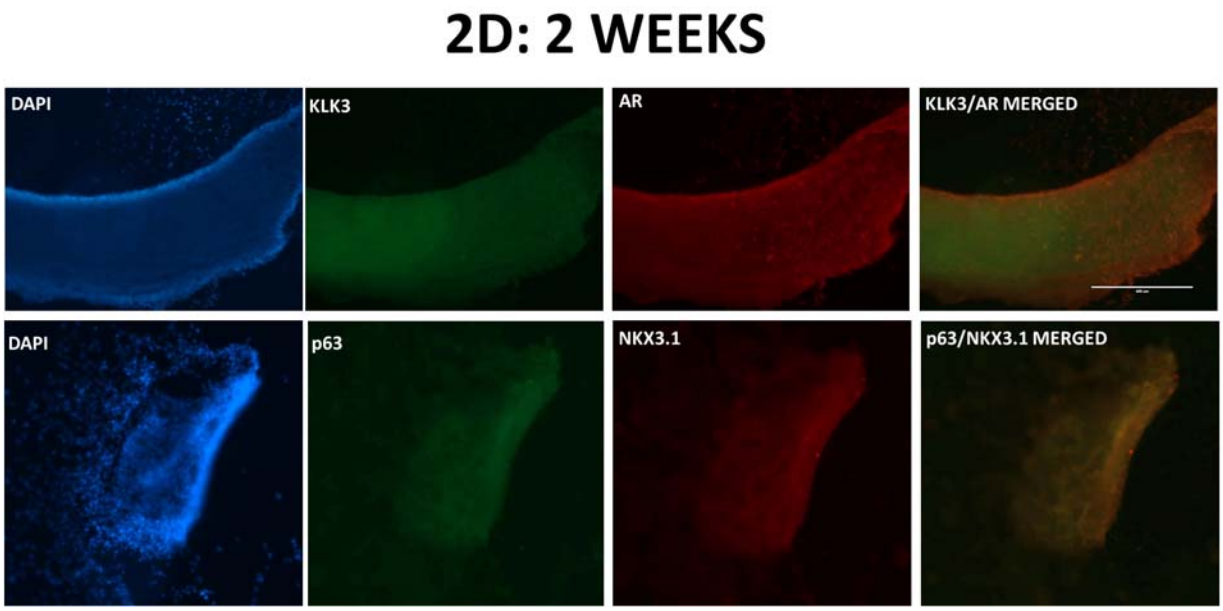

Figure 7. 2D Organoids Express Prostate Markers after 2 weeks. To evaluate the progress of the organoid diffentiation, we measured the expression of some prostate markers 2 weeks after differentiation in 2D organoids using immunofluorescence (IF). Results indicated that organoids showed early expression of KALLIKREIN 3 (KLK3)/ prostate specific antigen (PSA), androgen receptor (AR), prostate basal cell marker p63 and epithelial transcription factor NKX3.1.

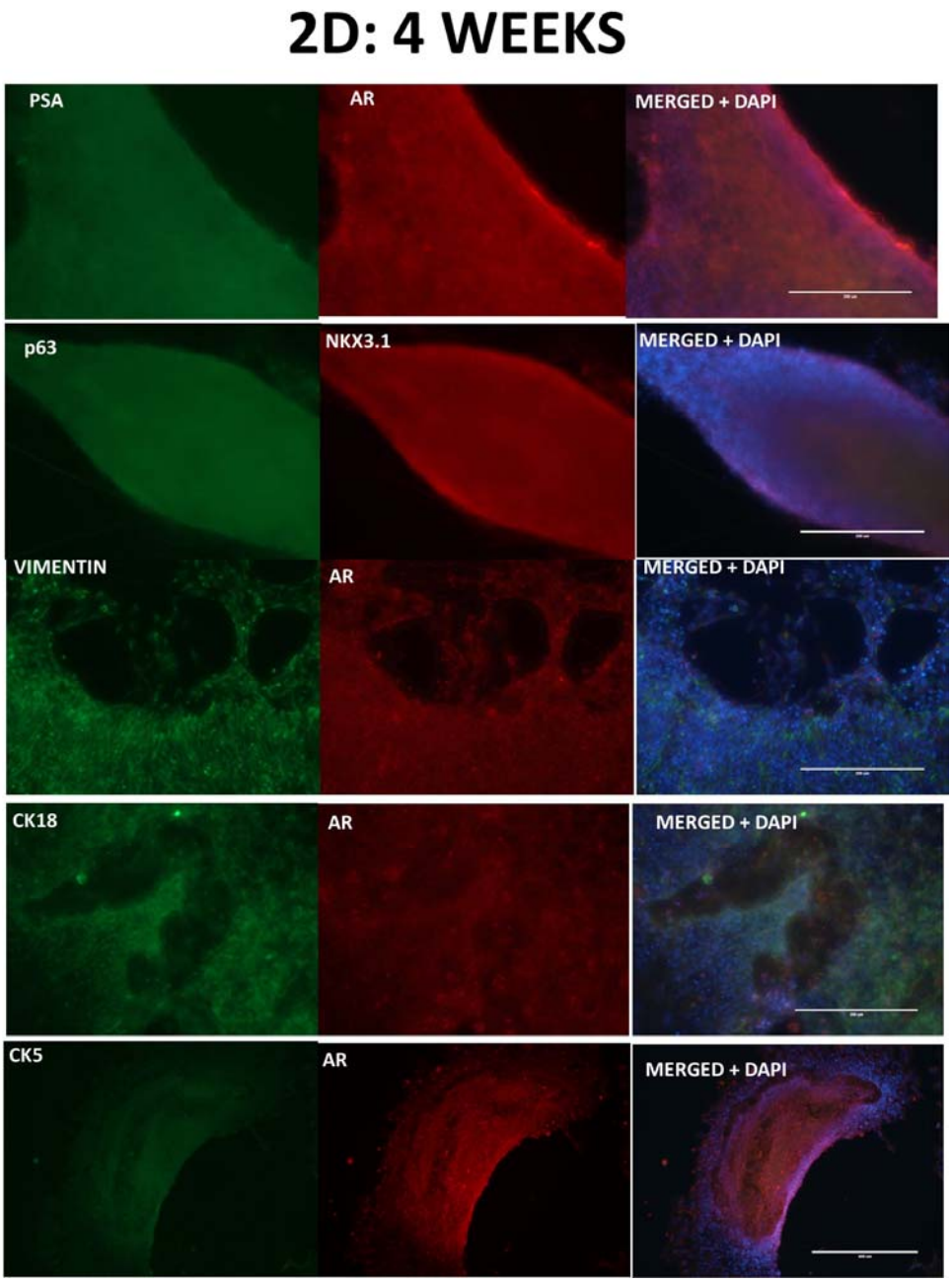

Figure 8. Prostate Markers expression increased after 4 weeks of differentiation of 2D organoids. We evaluated the differentiation process by determining the expression of prostate markers using immunofluorescence. We found that, 2D prostate organoids expressed the prostate specific antigen/KALLIKREIN3 (PSA/KLK3), androgen receptor (AR), prostate basal cell marker p63 and epithelial transcription factor NKX3.1 as early as two weeks after induction of prostate differentiation. We found that the expression of PSA/KLK3, AR, p63 and NKX3.1 increased after 4 weeks. Additionally, we measured the expression of epithelial luminal cell marker CYTOKERATIN 18 (CK18) and stromal cell marker VIMENTING, which were co-expressed with AR.

Expression of prostate markers was also determined in 3D organoids (Figure 9). After 4 weeks of prostate differentiation, 3D organoids were fixed and prepared for histological sections and staining. We found that consistent with 2D organoids, prostate markers were highly expressed in 3D organoids. Furthermore, we evaluated the expression of prostate markers in prostaspheres from 3D organoids. We collected and pellet the medium's supernatant from 3D organoids, to harvest prostatspheres fixation and prepared them for staining. Expression of prostate markers in prostaspheres was consistent with 2D and 3D organoids; in particular expression of KLK3/PSA, AR, p63, NKX3.1 and CK18. Altogether, these data demonstrate that iPSC-derived organoids display functional features similar to prostate organoids derived from adult prostate tissues.

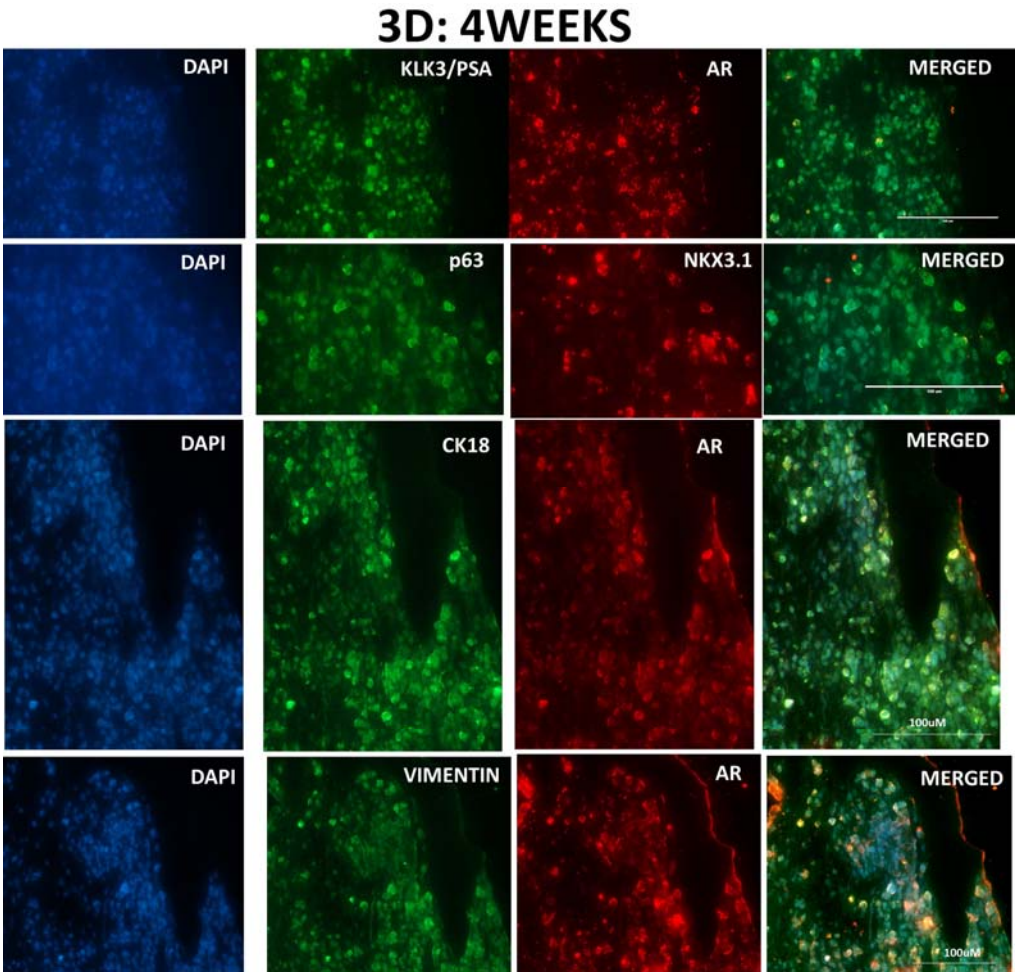

Figure 9. 3D organoids express Prostate Markers. Immunofluorescence staining revealed that, similar to 2D organoids, 3D organoid also expressed KLK3/PSA, AR, p63, NKX3.1, CK18 and VIMENTIN.

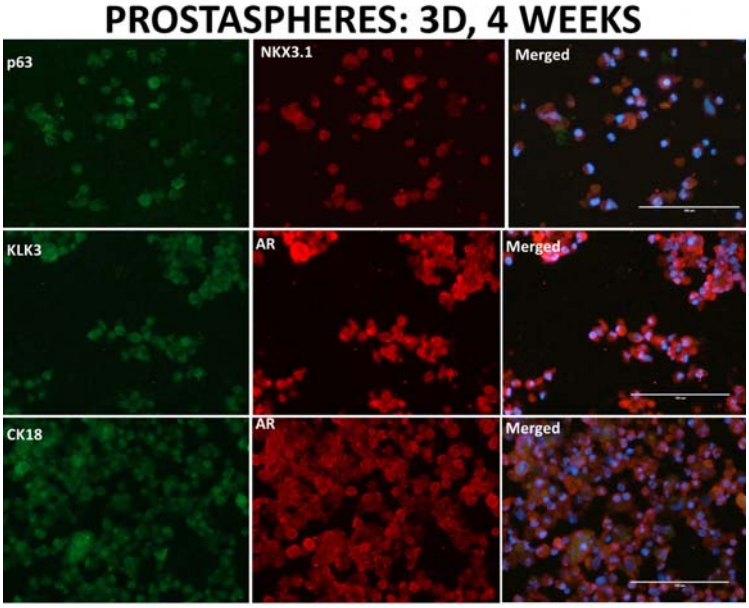

Figure 10. Prostaspheres express Prostate Markers. Similar to 2D and 3D organoids, prostaspheres generated by 3D prostate organoids expressed prostate markers.

We also analyzed the histological structure of the 3D organoids by H/E staining (Figure 11). Histological sections were evaluated by the UW-Skin Disease Research Center's experimental cutaneous pathology core specialists. This evaluation revealed that 3D organoids display stromal morphology with ductal branching, characteristics of prostate organs. Importantly, ducts regions show enriched differentiation, compared to other areas with more features that are stromal. Additionally, histology of longitudinal sections suggest that enriched areas are the active zones within the organoid's ducts that generate prostaspheres's budding. Cross sections also indicate that the organoids may form internal active zones with a primitive lumen-like morphology surrounded by stromal basal structure. H/E staining of prostaspheres collected from 3D organoid's shows a cellular nature of the budding structures, which is consistent with morphology of ducts' active zones and prostate markers expression determined by immunofluorescence.

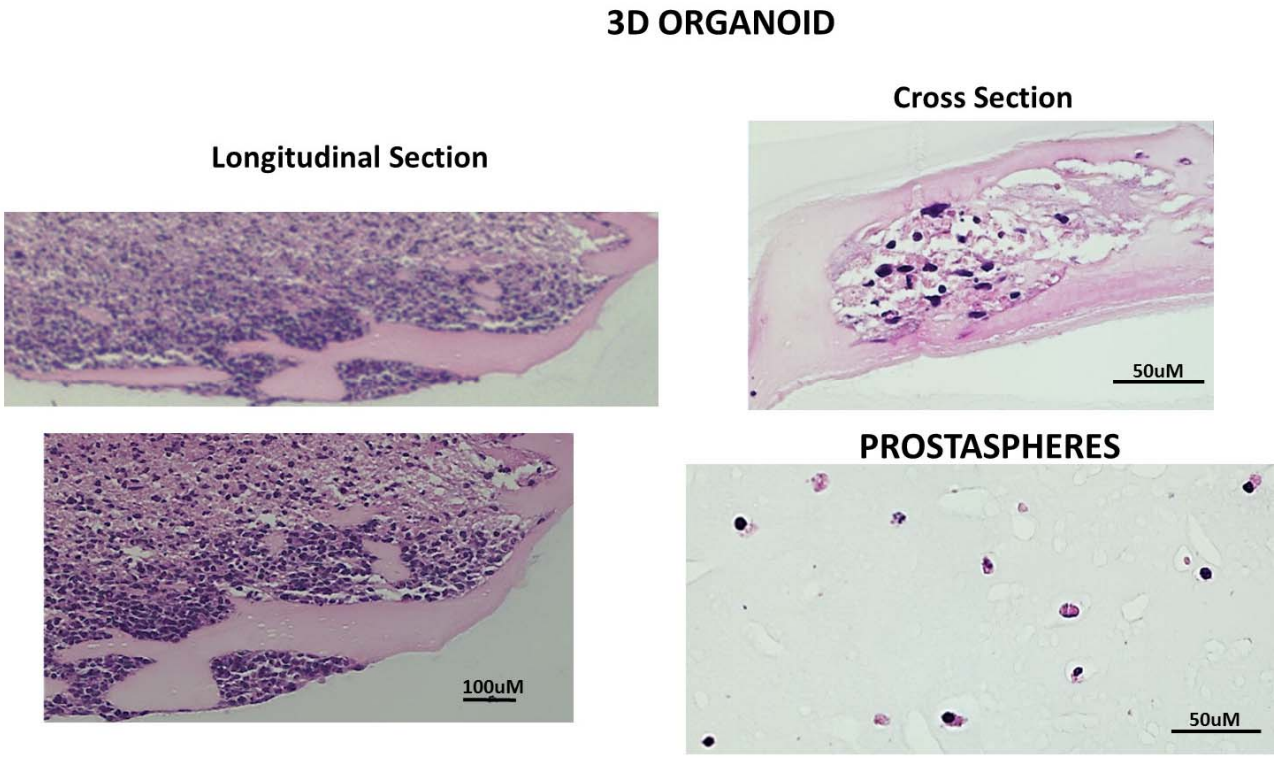

Figure 11. Prostate 3D Organoids display stromal cell-like organization with prostate duct branching and primitive luminal-like morphology. Histological analysis of 3D organoids was determined by H/E staining. Left panels display ducts branching and enriched differentiation. Cross section (top right) indicates that 3D prostate organoids may form internal active zones with a primitive lumen-like morphology surrounded by stromal basal structure. Prostaspheres' H/E staining (right bottom) shows their cellular nature with nuclei and cytoplasm, consistent with IF and with morphology of ducts' active zones.

- **What opportunities for training and professional development has the project provided?**
  - This project has further provided the opportunity for a postdoctoral fellow Dr. Edgardo Castro Perez to acquire skills in iPSC methodology, 2D and 3D organoids differentiation *in vitro* and learn concepts in prostate development and cancer.
- **How were the results disseminated to communities of interest?**
  - A manuscript is in preparation for publication in the month of November 2017.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Transcriptome and Epigenetic data from iPSC clones and iPSC-derived prostate organoids from European- and African- derived samples.
  - Experiments on potential genetic susceptibility disparities assays of European- and African- derived iPS cells differentiated into prostate organoids.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - A new method for iPSC differentiation into 2D and 3D prostate organoids *in vitro*.
- **What was the impact on other disciplines?**
  - Nothing to report yet.
- **What was the impact on technology transfer?**
  - Nothing to Report
- **What was the impact on society beyond science and technology?**
  - Nothing to Report for this period

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

We are comparing three methods of prostate differentiation of iPSC to prostate epithelial cells using specific signaling molecules with our originally described method using murine urinary genital stromal cells and their conditioned medium.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Our iPSC clones displayed 50% of chromosomal abnormalities and identification of high quality iPSC clones took longer than anticipated during karyotyping analyses.
  - Finding the best conditions during optimization of iPS cells differentiation into prostate organoids was more challenging than expected. However, after using three approaches/methods we were able not only to differentiate iPSC into prostate cells, but also into 3D organoids with functional features of prostate organoids including prostaspheres formation and ductal branching together with expression of prostate markers.
  - Unfortunately, due to unavailability of a key media from LONZA for prostate organoids

differentiation (prostate stromal cell growth media bullet kit) some DNA and transcriptome analyses have been delayed. This is the only media commercially sold for prostate stromal cell. Meanwhile, we are testing new conditions with some alternative options. Additionally, we are starting to create a GFP reporter for prostate differentiation by cloning the promoter from the KLK3/PSA (KALLIKREIN3/PROSTATE SPECIFIC ANTIGEN) gene into a lentivirus vector.

- **Changes that had a significant impact on expenditures**
  - Nothing to Report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to Report.
- **Significant changes in use or care of human subjects**
  - None
- **Significant changes in use or care of vertebrate animals.**
  - None
- **Significant changes in use of biohazards and/or select agents**
  - None

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**.
  - **Journal publications.**

    A manuscript is in preparation for publication within the next few weeks:
    Castro-Perez E, Jayanthy A, Setaluri V. **Reprogramming of Induced Pluripotent Stem Cells and *in vitro* Differentiation into Prostate Organoids under Defined Conditions.** Manuscript *In preparation 2017.*
  - **Books or other non-periodical, one-time publications.**
    - Nothing to Report.
  - **Other publications, conference papers, and presentations.**
    - Nothing report
- **Website(s) or other Internet site(s)**.
  - Nothing to Report
- **Technologies or techniques.**
  - Nothing to report.

- **Inventions, patent applications, and/or licenses**
  - Nothing to Report.
- **Other Products**
  - Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**
  -

Name:	**Project Role:**	**Researcher Identifier**	**Nearest person month worked:**	**Contribution to Project:**	**Funding Support:**
Vijayasaradhi Setaluri	PI	None	1.2	Overall project administration	This grant
Nihal Ahmad	Co-Investigator	None	0.36	Contributor, supplies PCa cell lines	This grant
Rupa Sridharan	Co-Investigator	None	0.6	Contributor, iPSC characterization: has provided guidance in reprogramming protocols	This grant
Edgardo Castro Perez	Postdoctoral researcher	None	12	Performed most of experiments to date	This grant
Kirthana Prabhakar	Postdegree intern	None	3	Experimental support to Dr. Perez	This grant
Murray Brilliant	PI, Marshfield Clinical Research Foundation subaward	None	0.18	Project oversight: genotyping of fibroblasts	This grant
Lynn Ivacic	Research Associate	None	0.3	Performed genotyping experiments to date	This grant
Terrie Kitchner	Research Coordinator	None	0.12	Coordinated institutional regulatory matters	This grant

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report
- **What other organizations were involved as partners?**
  - **Organization Name:** Marshfield Clinic Research Foundation
  - **Location of Organization:** Marshfield, WI
  - **Partner's contribution to the projec**t

- **Financial support:** NA
- **In-kind support**: NA
- **Facilities**: NA
- **Collaboration**: Genetic ancestry analyses
- **Personnel exchanges:** NA
- **Other.**

8. **SPECIAL REPORTING REQUIREMENTS** NONE
    - **COLLABORATIVE AWARDS:***N/A*
    - **QUAD CHARTS:** *N/A*
9. **APPENDICES:** *N/A*